Identification of Trop-2 as an oncogene and an attractive therapeutic target in colon cancers

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Abstract
The cell surface protein Trop-2 is highly expressed in a wide variety of epithelial cancers. In contrast, there is little or no expression of Trop-2 in adult somatic tissue. Because it is a cell surface protein that is selectively expressed in tumor cells, Trop-2 is a potential therapeutic target. However, whether Trop-2 is actively involved in tumorigenesis and whether its targeting for treatment would be effective have not been examined. Here, we show that Trop-2 expression is necessary for tumorigenesis and invasiveness of colon cancer cells, as both are inhibited when Trop-2 expression is suppressed by RNA interference. Conversely, ectopic expression of Trop-2 in colon cancer cells enhances their capacity for anchorage-independent growth and ectopic expression of Trop-2 in NIH3T3 cells is sufficient to promote both anchorage-independent growth and tumorigenesis. Importantly, we show that an antibody against the extracellular domain of Trop-2 reduces tumor cell invasiveness. Therefore, we have identified Trop-2 as an oncogene that has potential as a therapeutic target. Given the restricted expression of Trop-2 in normal tissue, anti–Trop-2 therapeutics would be predicted to have limited toxicity. [Mol Cancer Ther 2008;7(2):280–5]

Introduction
Trop-2 (also referred to as M1S1, TACSTD2, EGP-1) is a cell surface protein found to be highly expressed in diverse epithelial cancers (1–4) and its expression correlates with aggressive tumor behavior (5–7). Notably, in contrast to tumor cells, somatic adult tissues show little or no Trop-2 expression (8, 9). However, whether it has a functional role in cancer or is simply a biomarker has never been determined. If Trop-2 proves to be functionally important in tumorigenesis, given its accessibility to antibodies as a cell surface protein and its selective expression in tumor cells, it would be an attractive target for treatment.

There is little known about Trop-2 function. In one study, Trop-2 was found to be a substrate of protein kinase C when tested in vitro (10). In another study, it was shown that Trop-2, although structurally resembling other adhesion molecules, was unable to function as such (11). In a third study, an antibody against Trop-2 was shown to alter intracellular calcium levels (12). Because it is highly expressed in some developing tissues, Trop-2 has been suggested to play a role in morphogenesis (13); however, humans born with homozygous inactivating mutations in Trop-2 show only limited pathology (9). Finally, to our knowledge, the role of Trop-2 in tumorigenesis has never been addressed.

This study was designed to identify novel anticancer targets. We started by doing a microarray analysis comparing the gene expression profile of a highly tumorigenic colon cancer cell line with the gene expression profile of a low tumorigenic subclone and found several genes that were differentially expressed. Of these, Trop-2 stood out because its levels were dramatically increased in the highly tumorigenic cells compared with the levels in the subclone and Trop-2 levels are increased in certain aggressive cancers (6, 7). These findings led us to hypothesize that Trop-2 may promote tumorigenesis and therefore could serve as a therapeutic target.

We found that although Trop-2 is not essential for proliferation under standard tissue culture conditions, it is required for anchorage-independent growth and tumorigenesis of several different colon cancer cell lines. Conversely, we found that Trop-2 expression is sufficient to increase anchorage-independent growth in colon cancer cells and promote anchorage-independent growth and tumorigenesis in nontransformed cells. These findings identify Trop-2 as an oncogene. Importantly, we show that an antibody against Trop-2 can block the invasiveness of aggressive colon cancer cells in an in vitro assay, suggesting that targeting Trop-2 may have therapeutic utility.

Materials and Methods
Cell Culture
Hct-116, SW480, 293T, and NIH3T3 cells were obtained from the American Type Culture Collection. BE colon carcinoma cells were a gift from C.J. Marshall (Cancer Research UK, London, United Kingdom) and Hct-116-ltp
cells, a gift of P. Jallepalli (Memorial Sloan-Kettering Cancer Center, New York, NY), are a subclone of the Hct-116 cell line in which the gene securin has been deleted by homologous recombination (14). Cells were grown in DMEM containing 10% fetal bovine serum.

**Anchorage-Independent Growth and Tumor Assays**

Agar (0.6%; bottom agar) was added to six-well plates and allowed to solidify. Cells (1 × 10⁵) were resuspended in 1 mL of 0.3% agar (top agar) and overlayed onto the bottom agar. Cells were fed with top agar every 3 days, and after 4 weeks, colonies >2 mm in diameter were counted. Tumor assays were done by injecting 1 × 10⁶ SW480 or BE cells or 2.5 × 10⁶ NIH3T3 cells into the flank of 6-week-old male athymic (NCr-nu/nu) nude mice (National Cancer Institute, Frederick, MD). Mice were then evaluated for tumor formation at the indicated intervals using the formula for a semiellipsoid, 4/3πr³.

**Gene Expression Analysis**

Total RNA was purified from Hct-116 and Hct-116-ltp cells. After reverse transcription with an oligo(dT)-T7 primer, double-stranded cDNA was generated with the SuperScript Double-Stranded cDNA Synthesis kit (Invitrogen). Biotinylated cRNA was generated from the cDNA (Enzo Diagnostics) with T7 RNA polymerase (MessageAmp RNA kit, Ambion). Fragmented biotinylated cRNA was hybridized onto U133Plus arrays (Affymetrix) for 16 h at 45°C and the arrays were processed according to the manufacturer’s instructions. The arrays were then scanned with a high-numerical aperture and flying objective lens in the GS3000 scanner (Affymetrix). The image was quantified using GeneChip Operating Software 1.1 (Affymetrix) with the default variables for the statistical algorithm and all probe set scaling with a target intensity of 500.

**Flow Cytometry**

Trop-2 was detected with a monoclonal antibody (clone 162-46; Becton Dickinson) and an Alexa Fluor 488–conjugated anti-mouse secondary antibody (Invitrogen). Quantitation of surface Trop-2 expression was obtained using a Becton Dickinson FACSCalibur system. At least ten thousand events were analyzed by CellQuest software.

**RNA Interference and Retroviral Infections**

Short hairpins targeting human Trop-2 with the sequences 5′-CGTGGACAA CGATGGCCTCTA-3′ (nucleotides 906–926) and 5′-GCGCACGCTACATTCATACCT-3′ (nucleotides 1,377–1,397) were cloned into the lentiviral vector pLKO-puro (provided by S. Stewart, Washington University, Saint Louis, MO). BLAST analysis confirmed that both sequences are unique in the human genome. A hairpin targeting luciferase was used as a control (provided by S. Stewart). Lentivirus was generated as previously described (15). Infected cells were selected in puromycin to generate pooled stable knockdown populations. pBABE-puro-Trop-2 was generated by cloning the human Trop-2 cDNA between the EcoRI and BamHI sites in the pBABE-puro vector. After infection with pBABE-puro or pBABE-Trop-2, cells were selected in puromycin and the pooled populations were used in soft agar and tumorigenicity assays.

**In vitro Migration/Invasion Assays**

Costar 8-μm Transwell inserts were used for migration and invasion assays. Inserts were placed in a chamber of DMEM containing 10% FCS as a chemoattractant. BE cells (2 × 10⁵) were added directly to the insert in DMEM without serum. Cells were then allowed to migrate for 24 h. Unmigrated cells were removed from the top of the filter by swabbing and the remaining cells were fixed and stained with Hema3 (Fisher Scientific). The cells in five fields were counted for each group. For invasion assays, Matrigel (Becton Dickinson) without growth factors was diluted 1:3 in PBS, added to the upper chamber of the insert, and given time to solidify. Cells were added to the Matrigel and allowed to invade for 24 h. The filters were treated as described above and the invaded cells were scored as in the migration assays. Invasion is expressed as the ratio of the mean cell number of invaded cells to the mean cell number of migrated cells. For Trop-2 antibody blocking experiments, BE cells were incubated for 1 h with either control goat IgG or an anti–Trop-2 polyclonal antibody (R&D Systems) and assessed as above.

**Figure 1.** An Hct-116 subclone exhibits decreased anchorage-independent growth. A, colony formation in soft agar 2 wk after plating 1 × 10⁵ Hct-116 cells or Hct-116-ltp cells. B, quantitation of results from A. Columns, mean of three independent experiments; bars, SE.
**Results**

**Trop-2 Is Elevated in Highly Tumorigenic Colon Cancer Cells**

In an attempt to define new treatment targets, we identified a subclone of the Hct-116 human colon carcinoma cell line with low tumorigenic potential compared with the parental Hct-116 cells. We designated this subclone Hct-116-ltp (for low tumorigenic potential). Hct-116-ltp cells have a dramatically reduced ability to form colonies in soft agar when compared with the parental Hct-116 cells (Fig. 1A and B). When injected into the flanks of immunodeficient mice, Hct-116-ltp cells formed fewer tumors than Hct-116 cells (Table 1). Furthermore, the few tumors derived from Hct-116-ltp cells were on average <25% the size of tumors derived from Hct-116 cells when measured 4 weeks after xenografting (Table 1).

To begin to determine why Hct-116 cells are more tumorigenic than Hct-116-ltp cells, we compared the gene expression profiles of the two cell lines using cDNA microarrays. One of the largest differences between the two cell lines was a 17-fold decrease in Trop-2 mRNA levels in Hct-116-ltp cells compared with Hct-116 cells (Table 2). Trop-2 was also dramatically decreased at the protein level in the Hct-116 cells (Fig. 2A). These findings were notable because Trop-2 expression has been shown to correlate with tumor aggressiveness (6, 7) and it is not present in normal colonic epithelium (8, 9). Importantly, however, whether Trop-2 functions in tumorigenesis had not previously been determined.

**Trop-2 Is Important for Anchorage-Independent and Tumorigenic Growth**

To determine whether Trop-2 has a functional role in tumorigenesis, we suppressed its expression in colon cancer cells by RNA interference. We were unable to knock down Trop-2 expression in Hct-116 cells; however, we were successful in knocking down Trop-2 in two other colon cancer cell lines in which Trop-2 is highly expressed, SW480 and BE cells (Fig. 2B). In soft agar assays, Trop-2 knockdown cells formed <10% the number of colonies formed by control cells (Fig. 2C and D). Additionally, whereas tumors arose in 100% of the mice (eight of eight mice) injected with SW480 cells infected with the control hairpin, none of the mice (zero of eight mice) injected with the cells infected with a Trop-2 short hairpin developed tumors (Fig. 2E). BE cells also require Trop-2 to grow as tumors. Specifically, tumors arose in 100% of mice injected with BE cells harboring the control short hairpin (eight of eight mice), whereas no tumors developed in mice (zero of eight mice) injected with BE cells infected with a Trop-2 short hairpin. These results suggest that Trop-2 has a functional role in colon cancer.

**Trop-2 Is Oncogenic**

To further examine the role of Trop-2 in tumorigenesis, we first expressed Trop-2 in Hct-116-ltp cells. Trop-2 expression in these cells imparted the ability to form colonies in soft agar at nearly the same rate as Hct-116 cells (Fig. 3A and B). However, ectopic Trop-2 expression alone was insufficient to render Hct-116-ltp cells highly tumorigenic (data not shown), which is likely due to changes in the expression of other genes in these cells that are important for tumorigenesis. Indeed, when ectopically expressed in NIH3T3 cells, Trop-2 was sufficient to induce anchorage-independent growth, and it rendered these cells highly tumorigenic when injected into mice (Fig. 4A–C). NIH3T3 cells in which Trop-2 was ectopically expressed formed tumors in 100% of mice (n = 8) after s.c. injection. These findings provide further evidence that Trop-2 has a functional role in tumorigenesis.

**Statistical Analyses**

Comparison between two groups was done using the unpaired Student’s *t* test. Multiple comparisons were done by ANOVA.

**Table 1. Tumor frequency and volume in mice**

<table>
<thead>
<tr>
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<th>Tumors/injection</th>
<th>Mean tumor volume (cm³)</th>
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<tbody>
<tr>
<td>Hct-116</td>
<td>12/12</td>
<td>2.2 ± 0.6</td>
</tr>
<tr>
<td>Hct-116-ltp</td>
<td>3/12</td>
<td>0.5 ± 0.3</td>
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NOTE: Reduced frequency (*P* < 0.001) and volume (*P* < 0.05) in tumors derived from Hct-116-ltp cells compared with tumors derived from Hct-116 cells. Tumors were measured 4 wk after s.c. injection in mice. The mean tumor volume for Hct-116-ltp cells was calculated from the three tumors that formed. Mice received independent injections in both flanks.

**Table 2. Fold change in the 10 most significantly decreased genes in Hct-116-ltp cells compared with Hct-116 cells**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene name/function</th>
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<tr>
<td>17×</td>
<td><em>Trop-2</em> Tumor-associated calcium signal transducer 2</td>
</tr>
<tr>
<td>13×</td>
<td><em>NRIP1</em> Nuclear receptor interacting protein 1</td>
</tr>
<tr>
<td>8×</td>
<td><em>ZNF91</em> Zinc finger protein 91</td>
</tr>
<tr>
<td>7×</td>
<td><em>NSL1</em> MIND kinetochore complex</td>
</tr>
<tr>
<td>6×</td>
<td><em>ANXA10</em> Annexin 10</td>
</tr>
<tr>
<td>5×</td>
<td><em>MNS1</em> Meiosis-specific nuclear structural protein 1</td>
</tr>
<tr>
<td>4×</td>
<td><em>KIA</em> Inhibitor of differentiation 2</td>
</tr>
<tr>
<td>4×</td>
<td><em>FLJ34633</em> Unnamed open reading frame</td>
</tr>
<tr>
<td>4×</td>
<td><em>RG52</em> Regulator of G-protein signaling 2</td>
</tr>
<tr>
<td>3×</td>
<td><em>AREG</em> Amphiregulin</td>
</tr>
</tbody>
</table>

NOTE: The 10 genes most significantly decreased in Hct-116-ltp cells relative to their expression in Hct-116 cells as determined by microarray analysis. The results are expressed as fold decrease.
are important for tumorigenesis: anchorage-independent growth and invasiveness.

Finally, to begin to determine if Trop-2 could serve as an effective treatment target, we examined whether antibodies against Trop-2 block invasion. We found that BE cells treated with anti–Trop-2 antibodies were unable to invade through Matrigel (Fig. 5B). These results support the possibility of using anti–Trop-2 antibodies in the therapy of epithelial cancers in which Trop-2 is expressed.

Discussion

Trop-2 is expressed in a variety of cancers but not in the corresponding normal tissue; however, evidence that Trop-2 has a functional role in tumorigenesis has been lacking. We found that Trop-2 expression is required by a set of colon carcinoma cell lines for tumorigenic growth. Furthermore, we show that Trop-2 expression is required for invasion, and consistent with this observation, anti–Trop-2 antibodies can block colon cancer cell invasion. Conversely, we show that increasing Trop-2 expression increases anchorage-independent growth of colon cancer cells.

Figure 3. Trop-2 increases anchorage-independent growth of colon cancer cells. A, colony formation in soft agar 2 wk after plating $1 \times 10^5$ Hct-116 cells or Hct-116-ltp cells transduced with either pBABE-puro or pBABE-puro-Trop-2. B, quantitation of results from A. Columns, mean of three independent experiments; bars, SE.

Figure 2. Trop-2 suppression inhibits tumorigenesis. A, flow cytometry profiles of cell surface expression of Trop-2 on Hct-116 and Hct-116-ltp cells. B, flow cytometry profiles of cell surface expression of Trop-2 on SW480 and BE cells 5 d after infection with a lentiviral vector coding for a control or a Trop-2 short hairpin, shRNA, short hairpin RNA. C, colony formation in soft agar 2 wk after plating $1 \times 10^5$ SW480 cells harboring control or a Trop-2 short hairpin. D, quantitation of results from C. Columns, mean of three independent experiments; bars, SE. E, representative mice 4 wk after flank injection with $1 \times 10^6$ SW480 cells infected with a lentivirus coding for control or a Trop-2 short hairpin. Two different hairpins were used in each of the above experiments producing equivalent results. Additionally, knockdown of Trop-2 in BE cells produced similar results.
anchorage-independent growth of a colon cancer cell line and expression of Trop-2 transforms NIH3T3 cells. Collectively, these results clearly show that Trop-2 has a functional role in tumorigenesis.

Notably, Trop-2 expression has been associated with particularly aggressive tumors in a variety of cancers. For example, Trop-2 expression in primary colon tumors is associated with the development of liver metastases that are not curable by current medical therapies (7). In breast tumors, Trop-2 expression has been found to predict the development of lymph node metastases (5), which are the single most strongly predictive factor for reduced survival (18). In lung cancer, Trop-2 expression marks a subset of cells that are resistant to growth factor inhibitors (6). Therefore, Trop-2 is expressed in tumors for which new therapeutic approaches need to be developed.

Trop-2 is an especially attractive candidate for development as a therapeutic target. Its presence on the cell surface renders it accessible to antibody-based therapeutics. Furthermore, the restricted expression of Trop-2 in normal tissues predicts a high level of specificity and therefore limited toxicity for treatments that target Trop-2. Trop-2 resembles the Her2Neu oncogene in that both are highly expressed in tumor cells (19), show limited expression in normal tissues, and are cell surface proteins. Notably, Herceptin, an antibody used to treat Her2Neu-overexpressing breast tumors, dramatically increases survival and causes only limited toxicity (20). Anti–Trop-2 therapeutics could ultimately prove to be similarly useful against the subset of aggressive tumors in which Trop-2 is expressed.

Figure 4. Trop-2 is oncogenic. A, colony formation in soft agar 2 wk after plating 1 × 10⁵ NIH3T3 cells transduced with an empty vector or a vector containing a Trop-2 cDNA. B, quantitation of results from A. Columns, mean of three independent experiments; bars, SE. C, tumor formation in representative nude mice 4 wk after injection with Trop-2–expressing 2.5 × 10⁶ NIH3T3 cells. Mice injected with control cells showed no evidence of tumor formation when monitored for up to 12 wk.

Figure 5. Trop-2 is important for invasion. A, quantitation of the invasiveness of BE cells after Trop-2 suppression (P ≤ 0.05). B, quantitation of the invasiveness of BE cells after treatment with anti–Trop-2 antibodies (Ab) or IgG (P ≤ 0.05).
Acknowledgments
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References
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